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Inhibition of MAP/ERK kinase prevents IGF-I-induced hypertrophy in rat muscles

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Haddad, Fadia, and Gregory R. Adams. Inhibition of MAP/ERK kinase prevents IGF-I-induced hypertrophy in rat muscles. *J Appl Physiol* 96: 203–210, 2004. First published September 5, 2003; 10.1152/jappphysiol.00856.2003.—Insulin-like growth factor-I (IGF-I) has been shown to stimulate a hypertrophy response in skeletal muscles in vivo. In vitro studies have delineated two primary intracellular pathways that appear to mediate the effects of IGF-I in skeletal muscle: the Ras-ERK pathway and the phosphoinositide-3 kinase pathway. In vitro, the Ras pathway appears to regulate the mitogenic effects of IGF-I signaling, whereas the phosphoinositide-3 kinase pathway is associated with cellular differentiation. On the basis of the results from in vitro studies, we hypothesized that the coinfusion of both IGF-I and an inhibitor of the Ras pathway would result in some increase in muscle protein but an inhibition of cell proliferation. Our results show that 14 days of coinfusion of MAPK/ERK kinase inhibitor PD-098059 (PD) limited the phosphorylation of ERK and prevented IGF-I induced increases in protein (18%, $P < 0.05$ vs. 7%, not significant) or myofibrillar protein (23%, $P < 0.01$ vs. 5%, not significant). However, there were similar increases in indicators of cell proliferation (e.g., total DNA, 50 and 52%, $P < 0.001$) in both the IGF- and IGF+PD-infused muscles. The most notable impact on IGF-I signaling was a significant blunting of IGF-I induced increase in S6K1 phosphorylation by PD-98059 coinfusion (~5-fold, $P < 0.001$ vs. 3-fold, $P < 0.01$). These results suggest that there are interactions between the various pathways down stream of the IGF-I receptor that may behave differently in vivo than in myogenic cell lines in vitro.

insulin-like growth factor; p70 S6 kinase; cyclin D1; myogenin; insulin-like growth factor binding protein-5

SKELETAL MUSCLE RESPONDS TO chronic increases in mechanical loading with a compensatory hypertrophy. This hypertrophy response is specific to the muscles impacted by the increased loading, indicating that the adaptations are mediated by intrinsic mechanisms as opposed to more generalized somatic mediators (i.e., circulating hormones and growth factors). A consistent observation is that the compensatory hypertrophy process is accompanied by alterations in the expression of IGF-I and IGF binding proteins (IGFBP) in the affected muscles (1). More pointedly, IGF-I has been shown in numerous studies to have both mitogenic and anabolic effects on skeletal muscle and to induce skeletal muscle hypertrophy, establishing a clear cause-and-effect relationship (1).

Among the well-characterized growth factors, the ability to mediate both the proliferation and differentiation of myoblasts or muscle stem cells appears to be unique to IGF-I (35, 45). Analysis of the IGF type 1 receptor (IGFR1) indicates that the ability to promote cell differentiation can be dissociated from the mitogenic effects of IGFR1 ligation, suggesting that distinct intracellular signaling pathways may mediate these pro-

cesses (29). In vitro characterization of the intracellular signaling pathways that respond to the ligation of IGFR1 in skeletal muscle has lead to a model focused primarily on two pathways, one that is characterized by increased activity of Ras-ERK (extracellular signal-regulated kinase) cascade and a second that involves phosphoinositide-3 kinase (PI3K) signaling (13, 45). Coolican et al. (13) demonstrated that the dissociation of IGF-I-induced cell proliferation from cell differentiation could be attained via the inhibition of one or the other of these two pathways. In that study, Coolican et al. found that the Ras-ERK pathway was important for stimulating cell proliferation, whereas the PI3K pathway was linked to cell differentiation.

We have previously reported that the infusion of nonsystemic doses of IGF-I directly into the myofibrillar compartment of skeletal muscles results in hypertrophy (4). In the present study, we have infused IGF-I either alone or in combination with the MAPK/ERK kinase (MEK) inhibitor PD-098059. The results of this study indicate that intact signaling through MEK to the ERKs is necessary for the development of IGF-I-induced skeletal muscle hypertrophy in vivo.

METHODS

Sixteen female Sprague-Dawley rats weighing 206 ± 4 g were randomly assigned to two groups ($n = 8$). One group, designated IGF, received localized infusion of IGF-I into the plantaris muscle of one leg. A second group, designated IGF+PD, received an infusion of IGF-I and MEK inhibitor PD-098059 into the plantaris of one leg. IGF-I was infused at a rate of ~ 4 ng·mg muscle $^{-1}$ ·day $^{-1}$, a dose known to induce muscle hypertrophy in this model (4). In the coinfusion study, a 20 μ M concentration of PD-098059 was chosen on the basis of the methods reported for in vitro studies (e.g., Ref. 13).

Local infusion. Infusion was accomplished via a catheter attached to a miniosmotic pump [Alzet model 2002 (14 days), Alza]. The miniosmotic pumps were filled under aseptic conditions by following the manufacturer's instructions. Catheters consisted of ~ 8 cm of 0.006-in. ID Teflon (TFE, Cole-Parmer) tubing mated to the osmotic pump via several sizes of Tygon tubing (Fisher Scientific). The TFE tubing was fenestrated by using a 30-gauge hypodermic needle. For catheter implantation, the rats were anesthetized with ketamine and acepromazine (80 and 2 mg/kg, respectively), and incisions were made in the skin overlying the gastrocnemius muscle and on the back ~ 4 cm caudal from the neck. The catheter was passed between the two heads of the gastrocnemius to gain access to the plantaris. Two small cuts were made in fascia of the plantaris muscle by use of iris scissors. One cut was near the proximal end of the muscle, and the other was distal, near the tendon. The catheter was tunneled under the fascia of the plantaris and secured in place with sutures (4-0 Ethicon) that passed through the fascia and around the catheter at both the entry and exit points. The proximal end of the catheter was tunneled under the skin to the back incision. The catheter was filled with the same

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solution placed in the pumps, and the distal end of the catheter was closed by tying off with 2-0 suture. The proximal end of the catheter was then mated with the osmotic pump, which had been primed by preincubation in sterile saline at 37°C. The pump was then placed under the skin via the back incision, and both incisions were closed. At the termination of the infusion protocols, the osmotic pumps were removed, and any remaining infusate was aspirated via a syringe to verify that the pumps had functioned correctly.

On the basis of the specifications for the osmotic pumps, the total volume delivered per day was $10.8 \pm 0.48 \mu\text{l}$ ($\sim 0.45 \mu\text{l/h}$). On the basis of the findings of Sreter and Woo (48), the volumes infused per hour would represent $\sim 0.6\%$ of the extracellular space in rat plantaris muscles of this size.

Tissue collection. Fourteen days after the implantation surgeries, rats from the IGF and IGF+PD groups were killed via Pentosol euthanasia solution. The plantaris muscles of both the infused and contralateral leg were removed, weighed, and quick frozen between blocks of dry ice. These muscles were stored at -80°C for later analysis.

Determination of hypertrophy. After dissection, left and right medial gastrocnemius, plantaris, and soleus muscles were quickly weighed on an electronic scale before further processing. Plantaris muscle protein was determined from whole muscle homogenates by use of the biuret method (22). Total muscle protein was calculated from the product of the concentration and the wet weight of the muscle sample recorded at death. Total myofibrillar protein was determined as previously described (52) using the method of Solaro et al. (47). Briefly, muscle samples were weighed and then homogenized in ~ 20 volumes of an ice-cold solution containing 250 mM sucrose, 100 mM KCl, and 5 mM EDTA. The homogenate was washed (suspended then centrifuged at 1,000 g for 10 min) successively in three solutions: 1) 250 mM sucrose, 100 mM KCl, 20 mM imidazole, and 5 mM EDTA (pH 6.8); 2) 0.5% Triton X-100 and 175 mM KCl (pH 6.8); and 3) 150 mM KCl and 20 mM imidazole (pH 7.0). Each wash was repeated three times. The final pellet was resuspended in 10 volumes of 150 mM KCl (relative to extracted muscle weight). The myofibrillar protein concentration of this final solution was determined by using the biuret method (22).

DNA determination. DNA concentration was measured in whole muscle homogenates by using a fluorometric assay for the DNA binding fluorochrome bisbenzimidide H-33258 (Calbiochem, San Diego, CA). Calf thymus DNA was used as a standard (31).

Total RNA isolation. Total RNA was extracted from preweighed frozen muscle samples by using the TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the company's protocol, which is based on the method described by Chomczynski and Sacchi (11). Extracted RNA was precipitated from the aqueous phase with isopropanol and, after being washed with ethanol, dried and suspended in a known volume of nuclease-free water. The RNA concentration was determined by optical density at 260 nm (using an OD260 unit equivalent to 40 $\mu\text{g/ml}$). The muscle total RNA concentration is calculated on the basis of total RNA yield and the weight of the extracted muscle piece. The RNA samples were stored frozen at -80°C to be used subsequently in determining the specific mRNA expression by using relative RT-PCR procedures.

Reverse transcription. One microgram of total RNA was reverse transcribed for each muscle sample by using the SuperScript II RT from GIBCO BRL and a mix of oligo(dT) (100 ng/reaction) and random primers (200 ng/reaction) in a 20- μl total reaction volume at 45°C for 50 min, according to the provided protocol. At the end of the RT reaction, the tubes were heated at 90°C for 5 min to stop the reaction and then were stored at -80°C until used in the PCR reactions for specific mRNA analyses (see below).

PCR. A relative RT-PCR method using 18S as internal standard (Ambion, Austin, TX) was applied to study the expression of specific mRNAs of interest, including IGF binding proteins (IGFBP-4 and IGFBP-5), myogenin, and cyclin D1. Primers were purchased from

Life Technology, GIBCO. We have previously published the sequence for the various primers used for the specific target mRNAs (24). Note that for IGFBP-4, the primer sequence is based on the mouse X76066 sequence. These mouse primers were selected on the basis of regions that are highly similar to the human IGFBP-4 cDNA, and they proved to be effective with rat mRNA. All of the primers were tested for their compatibility with the alternate 18S primers.

In each PCR reaction, 18S ribosomal RNA was coamplified with the target cDNA (mRNA) to serve as an internal standard and to allow correction for differences in starting amounts of total RNA. For the 18S amplification, we used the Alternate 18S Internal Standards (Ambion), which yields 324-bp product. The 18S primers were mixed with competitors at an optimized ratio that could range from 1:4 to 1:10, depending on the abundance of the target mRNA. Inclusion of 18S competitors was necessary to bring down the 18S signal, which allows its linear amplification to the same range as the coamplified target mRNA (Ambion, Relative RT-PCR kit protocol).

For each specific target mRNA, the reverse transcription and PCR reactions were carried under identical conditions by use of the same reagent premix for all of the samples to be compared in the study. To validate the consistency of the analysis procedures, at least one representative from each group was included in each RT-PCR run.

One microliter of each RT reaction (0- to 10-fold dilution depending on target mRNA abundance) was used for the PCR amplification. The PCR reactions were carried out in the presence of 2 mM MgCl_2 by using standard PCR buffer (GIBCO), 0.2 mM 2-deoxynucleotide 5'-triphosphate, 1 μM specific primer set, 0.5 μM 18S primer/competitor mix, and 0.75 unit of *Taq* DNA polymerase (GIBCO) in 25- μl total volume. Amplifications were carried out in a Stratagene Robocycler with an initial denaturing step of 3 min at 96°C, followed by 25 cycles of 1 min at 96°C, 1 min at 55°C (55–60°C depending on primers), 1 min at 72°C, and a final step of 3 min at 72°C. PCR products were separated on a 2–2.5% agarose gel by electrophoresis and stained with ethidium bromide, and signal quantification was conducted by laser scanning densitometry, as reported previously (56). In this approach, each specific mRNA signal is normalized to its corresponding 18S. For each primer set, PCR conditions (cDNA dilutions, 18S competitor/primer mix, MgCl_2 concentration, and annealing temperature) were set to optimal conditions, so that both the target mRNA and 18S product yields are in the linear range of the semilog plot when the yield is expressed as a function of the number of cycles (8).

Phosphorylation state of intracellular signaling proteins. The phosphorylation state of the p70-S6 kinase (S6K1) and extracellular response kinase 1 and 2 (ERK1/2) were examined by immunoblotting using phosphospecific antibodies (Cell Signaling Technology, Beverly, MA) as reported previously (2, 24). The antibodies used detected changes in phosphorylation of residue Thr389 of S6K1 and Thr183 and Tyr185 of rat ERK2. In each case, phosphorylation at these sites is critical for increased activity in vivo (e.g., Refs. 37, 54). Muscle samples were extracted by homogenization in seven volumes of ice-cold *buffer A* (50 mM Tris-HCl pH 7.8, 2 mM potassium phosphate, 2 mM EDTA, 2 mM EGTA, 50 mM β -glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM DTT, 3 mM benzamide, 1 mM sodium orthovanadate, 10 μM leupeptin, 5 $\mu\text{g/ml}$ aprotinin, 200 $\mu\text{g/ml}$ soybean trypsin inhibitor, and 1 mM AEBSF) using a motor-driven glass pestle. The homogenate was immediately centrifuged at 12,000 g for 30 min at 4°C. The supernatant was immediately saved in aliquots at -80°C for subsequent use in immunoblotting. The supernatant protein concentration was determined by using the Bio-Rad protein assay with BSA as standard. Approximately 50 μg of supernatant proteins were subjected to SDS-PAGE (12.5% T), according to standard protocol (33), and then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P) using 10% methanol, 1 mM orthovanadate, 25 mM Tris, 193 mM glycine, pH 8.3. Phospho-ERK1/2 and phospho-S6K1 were detected by using phosphorylation state-specific antibodies (9204 and 910, Cell Signal-

ing Technology) and enhanced chemiluminescence method of detection (Amersham). Signal intensity was determined by laser scanning densitometry (Molecular Dynamics/Image Quant). For each specific antibody, all of the samples were run under identical (previously optimized) conditions, including the transfer on the membrane, the reaction with the first and secondary antibodies, washing conditions, enhanced chemiluminescence detection, and the film exposure. To ensure the consistency of this analysis, at least one representative sample from each group was included in each gel run and Western analysis. In addition, a positive control, provided by the antibody manufacturer, was run on each gel to allow for normalization. For each set of Western blotting and detection conditions, the detected signal was directly proportional to the amount of protein loaded on the gel over a range of 20–150 μ g (data not shown).

Statistical analysis. All values are reported as means $\pm$ SE. Differences between the four muscle sets (contralateral and treatment $\times$ IGF and IGF+PD) were determined by ANOVA with post hoc testing (Student-Newman-Keuls) using the PRISM software package (Graphpad). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

RESULTS

Infusion protocol. As seen in our laboratory's previous study (4), the local infusion protocol did not appear to impact tissue other than the targeted muscles. The body weight of rats in the IGF and IGF+PD groups increased to a similar degree (18 and 16% increase, respectively), and the masses of the soleus and medial gastrocnemius muscles, adjacent to the infused plantaris, were not different from those of the contralateral leg. For each of the parameters measured there were no differences between contralateral plantaris muscles from the IGF vs. IGF+PD groups. To simplify presentation, the contralateral data have been condensed into one value designated "Control" in the following sections. However, all statistical comparisons were made by using the distinct set of muscles (e.g., contralateral vs. infused) in the two treatment groups.

Muscle hypertrophy. Local infusion of IGF-I for 14 days resulted in a 19% increase in muscle wet weight and an 18% increase in the total protein content of the IGF but not the IGF+PD plantaris muscles (Fig. 1A, Table 1). Total protein concentration was unchanged in both treatment groups (Table 1). IGF-I infusion resulted in a 23% increase in myofibrillar protein content (IGF group) that was completely blunted by coinfusion of MEK inhibitor PD-098059 (Fig. 1B). The myofibrillar protein concentration of both the IGF and IGF+PD muscles was slightly decreased (Table 1).

Mitogenic indicators. The DNA content of the IGF and IGF+PD infused muscles increased $\sim$ 50% compared with the contralateral muscles (Fig. 2A). The concentration of DNA was also increased by both treatments (Table 1). Similarly, there was a significant increase in the expression and/or accumulation of cyclin D1 mRNA in the IGF and IGF+PD muscles (Fig. 2B).

Myogenic processes. The mRNA for myogenin, a putative marker of myogenic differentiation, increased significantly in the IGF+PD muscles, whereas this change was not significant after IGF-I infusion alone (Fig. 3).

Intracellular signaling. Infusion of IGF-I resulted in an approximate fivefold increase in the phosphorylation of ERKs 1 and 2 (Fig. 4), whereas the total ERK levels were unchanged vs. contralateral muscles (data not shown). Coinfusion (IGF+PD) completely blunted the increase in ERK1 phosphor-

Fig. 1. Local muscle infusion of IGF-I results in significant hypertrophy in the rat plantaris muscle. Fourteen days of IGF-I infusion resulted in significant increases in total (A) and myofibrillar (B) protein. These effects were blocked by the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor PD-098059 (PD). For all measures, there were no differences between the contralateral muscles from the two groups. Accordingly, the contralateral data have been combined as a single control value. All statistical analyses were conducted via ANOVA using all 4 muscles. * $P < 0.05$ vs. contralateral controls, # $P < 0.05$ vs. IGF.

ylation and significantly reduced the increase in ERK2 phosphorylation (Fig. 4B). Similar to ERK2, the phosphorylation of S6K1 was increased fivefold in the IGF and 2.8-fold in the IGF+PD muscles (Fig. 5). The infusion protocols did not impact the total amount of S6K1 (data not shown).

Total RNA. The total RNA content and concentration were increased 68 and 48%, respectively, compared with the contralateral muscle in the IGF-infused plantaris (Fig. 6, Table 1). Coinfusion of the PD inhibitor blunted this increase such that the changes in RNA content and concentration were not statistically different from the contralateral value.

IGF-I-related mRNA. IGF-I infusion resulted in a 48 and 63% increase in IGFBP-5 mRNA in the IGF and IGF+PD muscles, respectively (Fig. 7A). The mRNA for IGFBP-4 increased significantly (4-fold) in the IGF+PD but not IGF muscles (Fig. 7B).

DISCUSSION

The evidence that increased IGF-I is sufficient for the induction of a hypertrophy response in skeletal muscle appears to be very compelling. Experiments that include in vivo transfection, transgenic mice, or direct infusion have all demonstrated that increased IGF-I will induce muscle hypertrophy (4, 7, 12). There is a sizable body of evidence that indicates that IGF-I may also be necessary for compensatory hypertrophy (3, 14, 16, 57).

The mechanisms by which IGF-I may be mediating the development of muscle hypertrophy appear to have been well

Table 1. Muscle hypertrophy after infusion of IGF-I and IGF-I+PD

	IGF		IGF+PD	
	Contralateral	Infused	Contralateral	Infused
Muscle mass, mg/g body wt	1.02±0.03	1.22±0.03 (+19%, $P<0.001$)	1.00±0.02	1.07±0.03
Protein concentration, mg/g	273.3±3.5	261.4±2.2	264.9±7.1	259.2±3.1
Myofibrillar protein concentration, mg/g	158.9±3.0	142.9±2.2 (-10%, $P<0.05$)	157.6±4.5	146.2±2.6 (-7%, $P<0.05$)
DNA concentration, mg/g	1.17±0.03	1.43±0.09 (21%, $P<0.05$)	1.12±0.04	1.56±0.11 (39%, $P<0.01$)
RNA concentration, mg/g	0.70±0.02	1.05±0.15 (+50%, $P<0.05$)	0.72±0.02	0.94±0.06

Values are means ± SE. PD, MAPK/extracellular signal-regulated kinase inhibitor PD-098059.

characterized in vitro (20, 21, 45). The anabolic effects of IGF-I receptor ligation are thought to be mediated via a signaling cascade that includes PI3K (e.g., Refs. 13, 40). The mitogenic impacts of IGF-I-initiated signaling have been reported to be mediated primarily via the Ras-ERK signaling pathway, the activity of which appears to block myogenic differentiation (13, 28, 41).

In general agreement with the myogenic cell line literature, the results of the present study indicate that the hypertrophic effects of IGF-I on skeletal muscle require full activation of the ERKs. However, some aspects of our results appear to be at odds with the details of this process as delineated by the

majority of the data generated by using in vitro models (13, 40, 45). For example, we did not find a depression in IGF-I-induced cyclin D1 expression or in cell proliferation (i.e., total DNA) as a result of the PD-098059 coinfusion (e.g., Refs. 13, 26, 36). Chakravarthy et al. (10) found that satellite cells from mice that overexpress IGF-I in muscle demonstrate a decrease in p27^{KIP1} expression that allows them to remain mitotically active much longer than wild-type satellite cells. In contrast to results from myogenic cell lines and in apparent agreement with our in vivo study, in primary cell culture, proliferation of the transgenic satellite cells was not sensitive to PD-098059 but was prevented by the inhibition of PI3K signaling (10).

In the present study, the combination of IGF-I and PD-098059 resulted in enhanced expression of myogenin similar to the results found in myogenic cell lines such as L4A1 cells (13, 40, 45). Myogenin is a putative marker of myogenic cell differentiation in fully innervated muscles (9, 13, 17, 18, 32, 46, 58). The increase in the expression and/or accumulation of myogenin mRNA would seem to be a paradoxical finding in muscles that did not hypertrophy. However, there are a number of reports that indicate that the activity of the Ras-ERK pathway inhibits myoblast differentiation and the expression of myogenin (e.g., Refs. 5, 13, 33, 41, 55). In this context, the enhanced expression of myogenin mRNA with PD-098059 coinfusion may be a function of the disinhibition of differentiation-related signaling in the IGF-I stimulated muscles. Alternatively, the increase in myogenin mRNA seen in the PD-coinfused muscles could be a result of prolonged expression due to the failure of subsequent differentiation steps.

Fig. 2. Local muscle infusion of IGF-I results in significant increases in indicators of mitogenic activity in rat plantaris muscle. A: total DNA content of the IGF-I and the IGF+PD muscles was significantly increased vs. the contralateral controls. B: expression and/or accumulation of the mRNA for cyclin D1 was significantly increased in both the IGF and IGF+PD muscles. * $P < 0.05$ vs. contralateral controls (C).

Fig. 3. Local muscle infusion of IGF-I+PD but not IGF-I resulted in an increase in the expression and/or accumulation of the mRNA for myogenin. * $P < 0.05$ vs. contralateral control (C).

Fig. 4. Local muscle infusion of IGF-I results in significant increases the phosphorylation of ERKs 1 and 2 (A and B, respectively). Coinfusion of IGF-I and the MEK inhibitor PD-098059 blunted this response. * $P < 0.05$ vs. contralateral controls (C), # $P < 0.05$ vs. IGF. Inset: representative Western blot image for phospho-ERK1/2 detection. Both ERK 1 and ERK 2 are detected by use of the same phosphospecific antibody.

The role of ERK activity in skeletal muscle is still unclear. For example, ERK2 activity is reported to be required for cyclin D1 expression in muscle cell lines and thus promotes cell proliferation (reviewed in Ref. 36). However, there is evidence that increased ERK activity may also play a part in later differentiation processes (36). Sarbassov et al. (42) reported that higher ERK2-to-ERK1 phosphorylation ratios may be associated with myogenic differentiation, whereas the opposite ratio was associated with myogenic proliferation. In the present study, coinfusion of PD-098059 with IGF-I resulted in a mixed response, significantly increased cyclin D1 mRNA, and increased myogenin mRNA. In the IGF-I-infused muscles the bias was toward the proliferative response with increased cyclin D1 and no significant change in myogenin (Figs. 2 and 5). It is possible that the residual ERK2 activity, as indicated by phosphorylation state, may have been sufficient to stimulate cyclin D1 mRNA expression and cellular proliferation (Fig. 2), whereas the overall suppression of ERK phosphorylation disinhibited pathways leading to myogenin expression (Fig. 6).

The results of a number of studies have indicated that there is cooperativity between the ERK and PI3K pathway (6, 23, 38, 43, 44, 53). However, these relationships have not been clearly defined in skeletal muscle. For example, Samuel et al. (41) reported that treatment with PD-098059 resulted in in-

Fig. 5. Local muscle infusion of IGF-I and IGF+PD results in significant increases in the phosphorylation of p70 S6 kinase (S6K1). The increase in S6K1 phosphorylation was significantly lower than that seen with IGF-I alone. * $P < 0.05$ vs. contralateral controls, # $P < 0.05$ vs. IGF. Inset: representative Western blot image showing the phosphorylation state of S6K1.

creased S6K1 activity in L6A1 cells, indicating that MEK/ERK activity is inhibitory for signaling in the PI3K pathway. Kumar et al. (30) found that PI3K activity was required for the induction of increased ERK phosphorylation in response to axial stretching of the diaphragm muscle. Although it has been reported that an early step in the activation of S6K1 appears to be phosphorylation of COOH terminus regulatory sites by p38 or ERK (34), the results from the present study also suggest that ERK activation may have some positive impact on signaling events downstream from PI3K. According to this interpretation, the present findings may indicate that there are a number of direct or indirect interactions between various intracellular signaling pathways in skeletal muscles in vivo that modulate the impacts of ligation of the IGF-I receptor. Alternatively, the lesser increase in S6K1 phosphorylation in the PD-coinfused muscles may simply be a result of decreased IGF-I receptor ligation due to an increase in the presence of IGFBP-4 and thus not directly linked to ERK status.

Fig. 6. Local muscle infusion of IGF-I results in a significant increase in total RNA. The IGF-I-induced increase in RNA was not significant in the presence of the MEK inhibitor. * $P < 0.05$ vs. contralateral controls.

Fig. 7. Local muscle infusion resulted in alterations in the expression and/or accumulation of the mRNA for IGF binding proteins (IGFBP)-4 and IGFBP-5. **A:** mRNA for IGFBP-5 was significantly increased in both the IGF- and IGF+PD-infused muscles. **B:** local infusion of IGF+PD resulted in a significant increase in the mRNA for IGFBP-4. * $P < 0.05$ vs. contralateral controls, # $P < 0.05$ vs. IGF.

In general, the impact of IGFBP-4, relative to IGF-I activity, is thought to be inhibitory (19, 39). In the context of skeletal muscle, increased expression of IGFBP-4 has been associated with an inhibition of myogenic cell proliferation and/or differentiation in vitro (15, 18, 19). In the present study, we found that the levels of IGFBP-4 mRNA were significantly increased in muscles that were coin infused with IGF-I and the inhibitory PD compound (Fig. 7). Using cyclin D1 mRNA and DNA content as indicators, it does not appear that any increases in IGFBP-4 expression had an impact on cellular proliferation. However, the absence of a hypertrophy response to IGF-I may indicate that the presence of increased IGFBP-4 levels reduced ligation of the IGF-I receptor, therefore limiting anabolic processes. This result would be in accord with the lesser increase in S6K1 phosphorylation seen with PD coin fusion (Fig. 5).

The majority of the material detected in the RNA assay is ribosomal RNA. An increase in this value, as seen with IGF-I infusion, would therefore indicate an increase in translational capacity. Thus the lack of a significant increase in RNA concentration in the PD-coin fused muscles suggests that the

failure of these muscles to hypertrophy may be a result of an insufficient increase in translational capacity. Although it is somewhat controversial (49), the majority of the data suggest that S6K1 phosphorylation, and therefore activation, is important in mediating an increase in translational capacity by promoting the translation of key components of the translational machinery (25, 27, 50, 51). Therefore, the key mechanistic failure in the PD-coin fused muscles may have been the lesser increase in S6K1 phosphorylation.

It is difficult to directly equate the results of this study with those reported from in vitro work. For example, in the present study the treatments were continuous for 14 days with just a single time point analyzed. The work from Rosenthal's group has shown that the cellular response to IGF-I is biphasic in nature (e.g., Ref. 5). We have no way of knowing where our 14-day data fall on the temporal scale of such a biphasic response. Similarly, the whole muscle measurements provided in this work cannot differentiate between responses from various cell populations within the muscle.

In light of these limitations, there are several possible interpretations that could be made regarding our results. One interpretation would be that these results are, in some way, simply an artifact related to this particular experimental design. Obviously, we do not think that this is the high-probability interpretation. As we have previously reported, sham- or vehicle-infused muscles do not differ in any respect from untreated controls (Ref. 4, unpublished observations). In addition, we have consistently found that IGF-I infusion results in significant muscle hypertrophy (Ref. 4, unpublished observations; Fig. 1). Therefore, we feel that the coin fusion of PD-098059 was, in fact, impacting physiologically relevant IGF-I-induced responses. Accepting this assumption, the most logical interpretation of these results would be that the in vivo behavior of myofibers differs in some aspects from that seen in cell culture. This should not be a particularly surprising circumstance. The complex in vivo environment includes blood perfusion with all of the attendant mediators, such as growth factors, hormones, and nutritional signals. Similarly, myofibers experience neural inputs, cell-cell interactions, autocrine-paracrine signaling, and mechanical loading. Given these extensive differences between cell culture and in vivo conditions, it is actually quite surprising that there is often a high degree of correspondence between these two systems.

With regard to the specific mechanisms that may have been impacted in this study, the possibility of artifact is increased. Clearly the coin fusion of PD-098059 prevented IGF-I-induced hypertrophy. Similarly, the DNA and cyclin D1 data indicate that cell proliferation was not prevented in this model. However, more subtle differences, such as the increase in myogenin mRNA, could be a result of regional differences. For example, it is possible that myofibers more distant from the infusion site were at a different stage of response than those nearest the catheter when the tissue was sampled. We had previously reported that a gradient of infusate is found in tibialis anterior muscles using this method (4). However, we feel that this regional phenomenon is less likely in the present study, which used the smaller, more cylindrical, plantaris muscle.

A number of studies have demonstrated that cellular proliferation appears to be required for the development of significant compensatory hypertrophy in mammals. The results of the present study suggest that cell proliferation in the absence of

anabolic processes is not sufficient to induce hypertrophy. We had previously reported a similar finding, that local skeletal muscle infusion of FGF-2 results in increased cell proliferation but not muscle hypertrophy (4). Similarly, Miller et al. (35) reported that increased satellite cell proliferation, stimulated by hepatocyte growth factor, failed to promote muscle regeneration after muscle injury. Taken together, these results suggest that the processes of cell proliferation, differentiation, and fusion must be coordinated with anabolism to produce significant levels of muscle hypertrophy in response to IGF-I. As noted above, the dissociation of IGF-I-stimulated muscle cell proliferation from cell differentiation is not a unique observation (e.g., Refs. 5, 13, 29). However, the results of the present study would suggest that a more complex set of intracellular signaling interactions may occur in intact skeletal muscle in vivo compared with the conditions in cell culture.

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REFERENCES

- Adams GR. Invited Review: Autocrine/paracrine IGF-I and skeletal muscle adaptation. *J Appl Physiol* 93: 1159–1167, 2002.
- Adams GR, Caiozzo VJ, Haddad F, and Baldwin KM. Cellular and molecular responses to increased skeletal muscle loading after irradiation. *Am J Physiol Cell Physiol* 283: C1182–C1195, 2002.
- Adams GR and Haddad F. The relationships between IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy. *J Appl Physiol* 81: 2509–2516, 1996.
- Adams GR and McCue SA. Localized infusion of IGF-I results in skeletal muscle hypertrophy in rats. *J Appl Physiol* 84: 1716–1722, 1998.
- Adi S, Bin-Abbas B, Wu NY, and Rosenthal SM. Early stimulation and late inhibition of extracellular signal-regulated kinase 1/2 phosphorylation by IGF-I: a potential mechanism mediating the switch in IGF-I action on skeletal muscle cell differentiation. *Endocrinology* 143: 511–516, 2002.
- Azpiazu I, Saltiel AR, DePaoli-Roach AA, and Lawrence JC. Regulation of both glycogen synthase and PHAS-1 by insulin in rat skeletal muscle involves mitogen-activated protein kinase-independent and rapamycin-sensitive pathways. *J Biol Chem* 271: 5033–5039, 1996.
- Barton-Davis ER, Shoturma DI, and Sweeney HL. Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. *Acta Physiol Scand* 167: 301–305, 1999.
- Bickel CS, Slade JM, Haddad F, Adams GR, and Dudley GA. Acute molecular responses of skeletal muscle to resistance exercise in able-bodied and spinal cord-injured subjects. *J Appl Physiol* 94: 2255–2262, 2003.
- Brunetti A and Goldfine ID. Role of myogenin in myoblast differentiation and its regulation by fibroblast growth factor. *J Biol Chem* 265: 5960–5963, 1990.
- Chakravarthy MV, Abrahams TW, Schwartz RJ, Fiorotto ML, and Booth FW. Insulin-like growth factor-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3'-kinase/Akt signaling pathway. *J Biol Chem* 275: 35942–35952, 2000.
- Chomczynski P and Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
- Clemmons DR. Multiple hormones stimulate the production of somatomedin by cultured human fibroblasts. *J Clin Endocrinol Metab* 58: 850–856, 1984.
- Coolican SA, Samuel DS, Ewton DZ, McWade FJ, and Florini JR. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J Biol Chem* 272: 6653–6662, 1997.
- Czerwinski SM, Martin JM, and Bechtel PJ. Modulation of IGF-I mRNA abundance during stretch induced skeletal muscle hypertrophy and regression. *J Appl Physiol* 76: 2026–2030, 1994.
- Damon SE, Haugk KL, Birnbaum RS, and Quinn LS. Retrovirally mediated overexpression of insulin-like growth factor binding protein 4: evidence that insulin-like growth factor is required for skeletal muscle differentiation. *J Cell Physiol* 175: 109–120, 1998.
- DeVol DL, Rotwein P, Sadow JL, Novakofski J, and Bechtel PJ. Activation of insulin like growth factor gene expression during work induced skeletal muscle growth. *Am J Physiol Endocrinol Metab* 259: E89–E95, 1990.
- Dulciet A, Piette J, and Changeux JP. Influence of innervation of myogenic factors and acetylcholine receptor α -subunit mRNAs. *Neuroreport* 2: 25–28, 1991.
- Dupont-Versteegden EE, Murphy RJL, Houle JD, Gurley CM, and Peterson CA. Activated satellite cells fail to restore myonuclear number in spinal cord transected and exercised rats. *Am J Physiol Cell Physiol* 277: C589–C597, 1999.
- Ewton DZ, Coolican SA, Mohan S, Chernauek SD, and Florini JR. Modulations of insulin-like growth factor actions in L6A1 myoblasts by IGFBP-4 and IGFBP-5. *J Cell Physiol* 177: 47–57, 1998.
- Florini JR, Ewton DZ, and Coolican SA. Growth hormone and insulin like growth factor system in myogenesis. *Endocr Rev* 17: 481–517, 1996.
- Frost RA and Lang CH. Regulation of insulin-like growth factor-I in skeletal muscle and muscle cells. *Minerva Endocrinol* 28: 53–73, 2003.
- Gornall AG, Bardawill CJ, and David MM. Determination of serum proteins by means of the biuret reaction. *J Biol Chem* 177: 751–766, 1949.
- Gu M, Lynch J, and Brecher P. Nitric oxide increases p21(Waf1/Cip1) expression by a cGMP-dependent pathway that includes activation of extracellular signal-regulated kinase and p70(S6k). *J Biol Chem* 275: 11389–11396, 2000.
- Haddad F and Adams GR. Acute cellular and molecular responses to resistance exercise. *J Appl Physiol* 93: 394–403, 2002.
- Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L, and Ferrari S. Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. *J Biol Chem* 273: 14424–14429, 1998.
- Jones NC, Fedorov YV, Rosenthal RS, and Olwin BB. ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. *J Cell Physiol* 186: 104–115, 2001.
- Kawasome H, Papst P, Webb S, Keller GM, Johnson GL, and Gelfand EW. Targeted disruption of p70s6k defines its role in protein synthesis and rapamycin sensitivity. *Proc Natl Acad Sci USA* 95: 5033–5038, 1998.
- Khurana A and Dey CS. Subtype specific roles of mitogen activated protein kinases in L6E9 skeletal muscle cell differentiation. *Mol Cell Biochem* 238: 27–39, 2002.
- Kim JJ and Accili D. Signalling through IGF-I and insulin receptors: where is the specificity? *Growth Horm IGF Res* 12: 84–90, 2002.
- Kumar A, Chaudhry I, Reid MB, and Boriek AM. Distinct signaling pathways are activated in response to mechanical stress applied axially and transversely to skeletal muscle fibers. *J Biol Chem* 277: 46493–46503, 2002.
- Labarca C and Paigen K. A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* 102: 344–352, 1980.
- Lagord C, Leibovitch MP, Carpentier G, Leibovitch SA, and Martelly I. The kinase inhibitor iso-H7 stimulates rat satellite cell differentiation through a non-protein kinase C pathway by increasing myogenin expression level. *Cell Biol Toxicol* 12: 177–185, 1996.
- Lehtinen SK, Rahkila P, Helenius M, Korhonen P, and Salminen A. Down-regulation of transcription factors AP-1, Sp-1, and NF-kappa B precedes myocyte differentiation. *Biochem Biophys Res Commun* 229: 36–43, 1996.
- Martin KA and Blenis J. Coordinate regulation of translation by the PI 3-kinase and mTOR pathways. *Adv Cancer Res* 86: 1–39, 2002.
- Miller KJ, Thaloer D, Matteson S, and Pavlath GK. Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. *Am J Physiol Cell Physiol* 278: C174–C181, 2000.
- Naya FJ and Olson E. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Curr Opin Cell Biol* 11: 683–688, 1999.
- Payne DM, Rossomando AJ, Martino P, Erickson AK, Her JH, Shabanowitz J, Hunt DF, Weber MJ, and Sturgill TW. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J* 10: 885–892, 1991.

38. Piwien-Pilipuk G, Huo JS, and Schwartz J. Growth hormone signal transduction. *J Pediatr Endocrinol Metab* 15: 771–786, 2002.
39. Rajaram S, Baylink DJ, and Mohan S. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocr Rev* 18: 801–831, 1997.
40. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, and Glass DJ. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* 3: 1009–1013, 2001.
41. Samuel DS, Ewton DZ, Coolican SA, Petley TD, McWade FJ, and Florini JR. Raf-1 activation stimulates proliferation and inhibits IGF-stimulated differentiation in L6A1 myoblasts. *Horm Metab Res* 31: 55–64, 1999.
42. Sarbassov DD, Jones LG, and Peterson CA. Extracellular signal-regulated kinase-1 and -2 respond differently to mitogenic and differentiative signaling pathways in myoblasts. *Mol Endocrinol* 11: 2038–2047, 1997.
43. Scassa ME, Guberman AS, Varone CL, and Canepa ET. Phosphatidylinositol 3-kinase and Ras/mitogen-activated protein kinase signaling pathways are required for the regulation of 5-aminolevulinate synthase gene expression by insulin. *Exp Cell Res* 271: 201–213, 2001.
44. Shi Y, Hsu JH, Hu L, Gera J, and Lichtenstein A. Signal pathways involved in activation of p70S6K and phosphorylation of 4E-BP1 following exposure of multiple myeloma tumor cells to interleukin-6. *J Biol Chem* 277: 15712–15720, 2002.
45. Singleton JR and Feldman EL. Insulin-like growth factor-I in muscle metabolism and myotherapies. *Neurobiol Dis* 8: 541–554, 2001.
46. Smith CK, Janney MJ, and Allen RE. Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation rat skeletal muscle satellite cells. *J Cell Physiol* 159: 379–385, 1994.
47. Solaro J, Pang DC, and Briggs FN. The purification of cardiac myofibrils with Triton X-100. *Biochim Biophys Acta* 245: 259–262, 1971.
48. Sreter FA and Woo G. Cell water, sodium, and potassium in red and white mammalian muscles. *Am J Physiol* 205: 1290–1294, 1963.
49. Stolovich M, Tang H, Hornstein E, Levy G, Cohen R, Bae SS, Birnbaum MJ, and Meyuhas O. Transduction of growth or mitogenic signals into translational activation of TOP mRNAs is fully reliant on the phosphatidylinositol 3-kinase-mediated pathway but requires neither S6K1 nor rpS6 phosphorylation. *Mol Cell Biol* 22: 8101–8103, 2002.
50. Thomas G and Hall MN. TOR signaling and control of cell growth. *Curr Opin Cell Biol* 9: 782–787, 1997.
51. Thompson MG and Palmer RM. Signaling pathways regulating protein turnover in skeletal muscle. *Cell Signal* 10: 1–11, 1998.
52. Tsika RW, Herrick RE, and Baldwin KM. Interaction of compensatory overload and hindlimb suspension on myosin isoform expression. *J Appl Physiol* 62: 2180–2186, 1987.
53. Wang L, Gout I, and Proud CG. Cross-talk between the ERK and p70 S6 kinase (S6K) signaling pathways. MEK-dependent activation of S6K2 in cardiomyocytes. *J Biol Chem* 276: 32670–32677, 2001.
54. Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, and Avruch J. Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. *J Biol Chem* 273: 16621–16629, 1998.
55. Winter B and Arnold HH. Activated raf kinase inhibits muscle cell differentiation through a MEF2-dependent mechanism. *J Cell Sci* 113: 4211–4220, 2000.
56. Wright C, Haddad F, Qin A, and Baldwin KM. Analysis of myosin heavy chain mRNA expression by RT-PCR. *J Appl Physiol* 83: 1389–1396, 1997.
57. Yang S, Alnaqeeb M, Simpson H, and Goldspink G. Cloning and characterization of an IGF-1 isoform expressed in skeletal muscle subjected to stretch. *J Muscle Res Cell Motil* 17: 487–495, 1996.
58. Zhang P, Wong C, Liu D, Finegold M, Harper JW, and Elledge SJ. p21 CIP1 and p57 KIP2 control muscle differentiation at the myogenic step. *Genes Dev* 13: 213–224, 1999.